Studies on sperm storage in the vas deferens of the spinifex hopping mouse (*Notomys alexis*)

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The cauda epididymidis, with its relatively cool temperature (32–35°C), is considered to be the main site of sperm storage in male mammals. However, in the adult male spinifex hopping mouse, *Notomys alexis*, similar numbers of spermatozoa are found in the vas deferens to those in the cauda epididymidis. The present study shows that, unlike in the laboratory mouse in which spermatozoa of the vas deferens are found mainly in the epididymal region of the duct, spermatozoa in the hopping mouse are localized mainly to the middle and urethral regions of the vas deferens which lies in the inguinal and lower abdominal region of the body cavity. After ligation of the vas deferens close to its connection with the epididymis, many spermatozoa in the vas deferens retain the potential for motility for up to 2 weeks, indicating that the viability of spermatozoa is not compromised by being restricted to core body temperature. This urethral region of the vas deferens, in which spermatozoa reside, has a highly divergent structural organization compared with that of common laboratory rodents in which there is an expanded lumen with a network of epithelial folds. Ultrastructural observations of the cells lining the duct indicate that there are not any marked differences in morphology compared with the cells lining the duct in common laboratory murids, but the infoldings of the vas deferens of the hopping mouse are highly vascular which might facilitate supply of oxygen and nutrients to the spermatozoa residing in the lumen.

Introduction

As spermatozoa traverse the mammalian epididymis they develop the ability for forward progression and undergo surface changes that enable them to bind to, and fuse with, the plasmalemma of the oocyte, resulting in fertilization (Bedford, 1975; Orgebin-Crist \textit{et al.}, 1975). The epididymis, and in particular the cauda epididymidis, also acts as the main storage site for spermatozoa. In most species, the vas deferens, which passes from the cauda epididymidis to the urethra, is a highly muscular duct that functions mainly to propel spermatozoa from the cauda epididymidis to the urethra during sperm emission and ejaculation. However, it is evident that the vas deferens is far more than a passive conduit for spermatozoa. The ultrastructural characteristics of the lining epithelial cells indicate considerable secretory and endocytotic activity with a consequence that the luminal environment, within which the spermatozoa are found, is modified presumably to maintain their viability (Niemi, 1965; Flickinger, 1973; Hoffer, 1976; for reviews see Hamilton, 1975; Robaire and Hermo, 1988; Hermo \textit{et al.}, 2002).

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Fig. 1. Dissection of the reproductive tract of an adult male spinifex hopping mouse, *Notomys alexis*, showing that the vas deferens (VD) is narrow for the epididymal third of the duct, but expanded for the rest of its length, the urethral segment. The site of ligation in the present experiments is indicated by a single arrow. The double arrow indicates the point at which the vas deferens was severed after ligation for determination of sperm motility in the epididymal and urethral segments. Also indicated are the very small testes (T), cauda epididymides (CE), ventral prostate (VP) and penis (P). Scale bar represents 3 mm.

and temperature (18–25°C), and food and water were provided *ad libitum* (Breed, 1989; Peirce and Breed, 2001).

For the first series of experiments, after the animals were killed with carbon dioxide, the length of the vas deferens of isolated adult males was determined with vernier callipers, and the duct was then divided into three segments of equal length. Some of the segments of the vas deferens were embedded in paraffin wax, sectioned and stained with either haematoxylin–eosin or Mallory's and/or Masson's trichrome for light microscopy. Spermatozoa were extruded from segments of other vasa deferentia, together with those from the cauda epididymidis, into 0.2 ml PBS. A haemocytometer was used to determine the total number of spermatozoa in the three regions of the vas deferens, and in the cauda epididymidis. Vasa deferentia from nine laboratory mice were also divided in a similar way and the relative abundance of spermatozoa was determined in each of the three segments of the vasa deferentia and in the cauda epididymidis.

### Ligation experiments

These experiments were designed so that each animal acted as its own control. Each adult, sexually rested, male hopping mouse was anaesthetized with halothane and a 3–5 mm segment of the vas deferens ligated near its connection with the cauda epididymidis (Fig. 1), taking care not to disturb the blood supply. The contralateral vas deferens was not ligated and acted as a control. The animals were allowed to recover, and five animals were assigned randomly to each of three groups. One group of five was killed by administration of an overdose of sodium pentobarbitone (Nembutal, Abbot Ceva Chemicals, Hornsby, NSW) at 1, 2 and 4 weeks after surgery. Data from two animals from the week 1 group and from one animal from the week 2 group were excluded from the results due to infection after the operation. The numbers of spermatozoa from the experimental and control vasa deferentia were compared by paired *t* tests, and the percentage of motile spermatozoa was estimated. For purposes of comparison with the hopping mice, ligation of the vas deferens, just distal to its connection with the cauda epididymidis, was also performed on four randomly bred laboratory mice. These animals were killed 1 week later and the percentage of motile spermatozoa in the vas deferens was determined.

The cauda epididymides and vasa deferentia of the hopping mice were removed post mortem. The vas deferens was severed at the region where the expansion occurred (Fig. 1), and numbers of spermatozoa in the cauda epididymides, and the epididymal and urethral regions of the vasa deferentia were determined separately. For this procedure, each segment of the duct was minced in physiological saline to release spermatozoa

### Materials and Methods

Sexually mature male spinifex hopping mice, *Notomys alexis*, were obtained from a colony bred at the Medical School Animal House, University of Adelaide, or from a local supplier. The mice were housed under conditions described previously in which there was regulated lighting (12 h light:12 h dark photoperiod).
from all the tissue fragments. The percentage of motility of each sperm suspension was estimated and the numbers of spermatozoa were determined using a haemocytometer.

In a second series of experiments, seven adult male hopping mice were anaesthetized with halothane and a ligature was placed proximal to the cauda epididymidis, at its junction with the corpus epididymidis. Again, animals were allowed to recover and were then killed either 1 or 2 weeks later, at which time the percentage of motile spermatozoa retained in the cauda epididymides and vasa deferentia was determined.

For electron microscopy, ten adult male hopping mice were anaesthetized with pentobarbitone sodium and fixed by perfusion via the left ventricle of the heart with transmission electron microscopy fixative (3% (w/v) formaldehyde and 3% (w/v) glutaraldehyde, pH 7.2) for 10 min, after first flushing the vascular system with a rinse solution (Forssmann et al., 1977). After initial fixation, the cauda epididymides and vasa deferentia were removed and each vas deferens was subdivided into epididymal and urethral portions at the point where the diameter of the duct increased (Fig. 1). Each of these regions was then cut into 2–3 mm pieces and immersed in EM fixative for a further 2–4 h at room temperature. The tissues were washed in 0.2 mol phosphate buffer l−1, fixed in 1% (w/v) OsO4 in 0.2 mol phosphate buffer l−1, rinsed in 0.2 mol phosphate buffer l−1 and stained en bloc with 1% (w/v) uranyl acetate in 0.1 mol maleate buffer l−1 for 1.5 h in the dark. The tissues were then dehydrated by passage through a series of increasing concentrations of ethanol and embedded in TAAB TK3 epoxy resin (TAAB Laboratories, Berkshire). Survey sections (0.5–1.0 μm) were cut, stained with 0.025% (v/v) toluidine blue in 0.5% (w/v) sodium tetaborate buffer and viewed with an Olympus BH-2 or Vanox microscope. For transmission electron microscopy, areas of interest were selected from survey sections; ultra-thin sections were cut, mounted onto copper–palladium grids and stained with saturated uranyl acetate in 70% (v/v) alcohol, followed by lead citrate. The grids were examined with a Philips CM100 transmission electron microscope.

**Results**

The present observations confirm previous findings that the vas deferens of the hopping mouse has a highly unusual morphology (Fig. 1). The duct is about 27 mm in length overall and is composed of two anatomically distinct regions. The epididymal region has a narrow duct surrounded by a thin muscle coat and is approximately 10 mm in length, and has an outer diameter of about 0.5 mm (Fig. 2a). The urethral section of the duct, which makes up two thirds of the vas deferens, has a significantly greater outer diameter of 1.3–1.7 mm (Fig. 1). Internally the urethral section of the vas deferens has numerous epithelial infoldings that are supported by a thin lamina propria (Figs 2b,c). Rather than a thick muscle coat as in most species, the external muscle wall is very thin, with the consequence that the duct is transparent and its contents can be seen to contain yellowish-white fluid. The thickness of the muscle layer is similar to that of the epididymal segment and is composed of only about ten layers of circular muscle and two to four layers of longitudinal smooth muscle cells (Breed, 1986; Peirce and Breed, 1989) (Fig. 2b,c).

When the numbers of spermatozoa were determined for the three equal segments of the vas deferens, it was found that in the laboratory mouse an average of 56% (range 46–77%) of the total vas deferens spermatozoa (n = 9) were concentrated in the epididymal segment of the duct with only a small percentage of spermatozoa in the urethral section of the vas deferens (Table 1). By contrast, in the hopping mouse, there was only about 15% of the vas deferens sperm population (range 3–27%, n = 8) in the epididymal region of the duct. In the middle segment of the vas deferens, which included the section where the diameter of the duct increased markedly and epithelial infoldings became apparent, an average of 35% (range 20–47%) of the total vas deferens sperm population was present, whereas in the urethral section of the vas deferens, an average of 47% of the total vas deferens spermatozoa was present (Table 1; Fig. 2b,c).

**Numbers and motility of spermatozoa after ligation of the vas deferens**

Data on the number and motility of spermatozoa recovered from the cauda epididymides and the epididymal and urethral portions of the vas deferens, after ligation of the vas deferens adjacent to the cauda epididymides are shown (Table 2). The number of spermatozoa within the cauda epididymides on the ligated side increased steadily throughout the subsequent 4 week period and the percentage of spermatozoa that developed motility was similar to that of the controls for the first 2 weeks after ligation, after which there was a small decline.

The number of spermatozoa in the epididymal region of the vas deferens distal to the ligation site at 1 week after ligation was similar to that in controls (P = 0.28), whereas in the urethral region of the duct, significantly fewer spermatozoa were present in the ligated vasa deferentia (paired t test, t = 3.26; P = 0.04). In all four laboratory mice killed 1 week after ligation of the vas deferens, there were no motile spermatozoa in the vas deferens distal to the ligation site, whereas in the hopping mouse, the percentage of motile spermatozoa was similar between the ligated and control vasa deferentia for both the proximal and distal segments of the duct. By 2 weeks after ligation, the number of spermatozoa within both of these regions of the vas deferens on the ligated
Fig. 2. For legend see facing page.
Table 1. Numbers and distribution of spermatozoa in the vas deferens of the spinifex hopping mouse (Notomys alexis) and the laboratory mouse

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of animals (n)</th>
<th>Number of spermatozoa in cauda (x10^6)</th>
<th>Number of spermatozoa in vas deferens (x10^6)</th>
<th>Percentage distribution of spermatozoa in the vas deferens (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hopping mouse</td>
<td>8</td>
<td>0.92 ± 0.25</td>
<td>1.15 ± 0.39</td>
<td>15 (3–27) 35 (20–47) 47 (38–58)</td>
</tr>
<tr>
<td>Laboratory mouse</td>
<td>9</td>
<td>17.86 ± 1.92</td>
<td>4.14 ± 0.79</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Effect of ligation of the vas deferens adjacent to the cauda epididymidis on the numbers and motility of spermatozoa within the excurrent ducts of the spinifex hopping mouse (Notomys alexis)

<table>
<thead>
<tr>
<th>Time after ligation (weeks)</th>
<th>Ligated side</th>
<th>Control side</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of spermatozoa x 10^5 (range)</td>
<td>Percentage motility of sperm population (range)</td>
</tr>
<tr>
<td>1 week^a</td>
<td>Cauda epididymidis 31.7 (11.0–52.0) 53 (50–60)</td>
<td>8.7 (6.2–12.0) 70 (70)</td>
</tr>
<tr>
<td></td>
<td>Epididymal portion of vas deferens 5.4 (3.5–8.2) 50 (50)</td>
<td>6.8 (6.1–7.8) 60 (50–70)</td>
</tr>
<tr>
<td></td>
<td>Urethral portion of vas deferens 0.7 (0.3–1.1) 50 (50)</td>
<td>1.7 (0.8–2.2) 57 (50–60)</td>
</tr>
<tr>
<td>2 weeks^b</td>
<td>Cauda epididymidis 92.5 (58–118) 63 (50–70)</td>
<td>13.2 (8.2–17.0) 64 (50–75)</td>
</tr>
<tr>
<td></td>
<td>Epididymal portion of vas deferens 2.4 (0.8–4.3) 33 (10–50)</td>
<td>8.5 (3.6–10.4) 58 (50–70)</td>
</tr>
<tr>
<td></td>
<td>Urethral portion of vas deferens 0.9 (0.3–1.6) 20 (10–30)</td>
<td>3.8 (2.7–6.6) 38 (30–50)</td>
</tr>
<tr>
<td>4 weeks^c</td>
<td>Cauda epididymidis 164.5 (129–198) 38 (30–50)</td>
<td>17.4 (11–24) 56 (50–70)</td>
</tr>
<tr>
<td></td>
<td>Epididymal portion of vas deferens 1.0 (0.4–1.8) 14 (5–30)</td>
<td>8.3 (3.8–12.0) 50 (40–60)</td>
</tr>
<tr>
<td></td>
<td>Urethral portion of vas deferens 0.1 (0.1) 1 (1)</td>
<td>3.1 (2.1–5.2) 50 (30–60)</td>
</tr>
</tbody>
</table>

^a n = 3 for all values; ^b n = 4 for all values; ^c n = 5 for all values. The vas deferens was severed at the point where expansion began (see Fig. 1) so that the number of spermatozoa in the epididymal and urethral sections could be assessed separately.

Ultrastructure of the vas deferens epithelium

The epithelium of the vas deferens is composed of a lining of principal cells and occasional basal cells. Transmission electron microscopy of the principal cells in the proximal region of the vas deferens showed that long stereocilia protrude into the lumen. In the apical region of the cells, many vesicles were often present. Mitochondria were also abundant, and there were a few spermatozoa distal to the ligated site, whereas in the two animals killed 2 weeks after ligation, 20 and 55% of the spermatozoa in the cauda epididymides were motile.

Ligation proximal to the cauda epididymidis

Two animals were excluded from the analysis due to adhesions. Of the six remaining animals, the four killed 1 week after ligation had between 20 and 60% motile spermatozoa distal to the ligated site, whereas in the two animals killed 2 weeks after ligation, 20 and 55% of the spermatozoa in the cauda epididymides were motile.

Fig. 2. Light micrographs of vas deferens from adult male spinifex hopping mice, Notomys alexis. (a) Light micrograph of a haematoxylin–eosin stained section taken from the region where the epididymal segment of the duct starts to enlarge and epithelium invaginations begin; note spermatozoa (SP) in the lumen. (b) Light micrograph of a haematoxylin–eosin stained section of the middle region of the vas deferens showing epithelial infoldings (EF) and abundant luminal spermatozoa (SP). (c) Light micrograph of a trichrome-stained section of the distal region of the vas deferens showing elaborate epithelial infoldings (EF) and abundant spermatozoa (SP) in the lumen. EP: epithelial layer; M: muscle layer. Scale bars represent 100 μm.
multivesicular bodies and lysosomes. Golgi complexes were very large (Fig. 3), whereas the few lysosomes generally showed heterogeneity. The nuclei tended to be basally located and there was an abundance of cisternae of rough endoplasmic reticulum around and basal to the nuclei.

In the expanded urethral section of the vas deferens, the principal cells lining the epithelial infoldings differed markedly in ultrastructure from those lining the epididymal region of the duct. Light microscopy showed that the nuclei were centrally, rather than basally, located (Figs 2b,c,4a) and that the underlying lamina propria was highly vascular (Fig. 4a). Many epithelial principal cells had cytoplasmic extensions that protruded into the lumen (Fig. 4b), from which stereocilia extended, some of which were branched. Coated pits and vesicles were either sparse or absent. Mitochondria were generally very abundant throughout the apical cytoplasm and there were some multivesicular bodies and occasional dense bodies in the perinuclear region but the Golgi complexes were not as extensive, or elaborate, as those in the principal cells of the proximal portion of the vas deferens. Some cisternae of rough endoplasmic reticulum occurred lateral to the nuclei, whereas, basally, abundant cisternae of smooth endoplasmic reticulum were evident.

Discussion
In nearly all species of mammal, the cauda epididymidis is the main extratesticular storage site for spermatozoa. However, in the spinifex hopping mouse, Notomys alexis, small numbers of spermatozoa are present in this region compared with species in other murid rodent genera including those in the laboratory mouse. In the hopping mouse, the cauda epididymidis, like the testis, is small, and, within this region, there are not only relatively few spermatozoa but also their numerical density within the lumen is low (Breed, 1997). However, in contrast to most other species, up to 60% of the total extratesticular sperm population resides within the vas deferens (Breed, 1986, 1997; Peirce and Breed, 1989). In the present study, the relative number of spermatozoa in three equal segments of the vas deferens was determined. Unlike the laboratory mouse, in which most spermatozoa of the vas deferens reside in the epididymal segment, in the hopping mouse, most of the sperm population of the vas deferens was located within the middle and urethral segments of the duct.

Ligation experiments showed that spermatozoa in the vasa deferentia of the laboratory mouse were no longer viable after being restricted to that region for 1 week, whereas many hopping mouse spermatozoa were motile when retained in the vas deferens for 1, or even 2, weeks. In general, this percentage was similar to that of the cauda epididymal sperm population after ligation of the duct at the corpus–cauda junction. Thus, these observations clearly support the view that the middle and distal regions of the vas deferens of the hopping mouse act as an accessory storage reservoir for functional spermatozoa. This region lies within the inguinal and pelvic regions of the abdominal cavity.
which has a temperature of about 37°C (Breed and Setchell, 2000), whereas the temperature of the scrotum is about 35.6°C. Storage of a large percentage of the extratesticular spermatozoa in this region is in contrast to observations in the laboratory rat and rabbit in which the sperm storage potential of the cauda epididymidis, when placed in the lower abdominal region of the body, is markedly depressed (Bedford, 1978; Foldesy and Bedford, 1982). Therefore, the hopping mouse appears to have evolved a strategy for retaining spermatozoa within the vas deferens at core body temperature for significant periods that does not have a deleterious effect on their potential for motility and, thus, probable fertilizability.

Peirce and Breed (2001) showed that, compared with common laboratory rodents (Aman et al., 1976; Robb et al., 1978; Dadonne and Alfonsi, 1984), spermatozoa of the hopping mouse have a faster transit time along the epididymis and epididymal sperm stores are relatively low. Consequently, many of the spermatozoa may pass rapidly from the epididymis to the vas deferens storage region where their final maturation may take place. Why this accessory sperm storage site has evolved in the hopping mouse remains an enigma, but it does not appear to be due to the lack of the storage capacity of the cauda epididymidis as the concentration of spermatozoa in this region is low compared with that of common laboratory rodents.

In the middle and urethral regions of the vas deferens, where storage of spermatozoa occurs, there is a highly elaborate, and convoluted, epithelial lining which is markedly different from the small lumen surrounded by a thick muscle coat of the urethral vas deferens in the common laboratory rats and mice. The principal cells of this region show similar ultrastructural features to those of laboratory rats and mice (Flickinger, 1973; Hamilton, 1975; Hamilton and Cooper, 1978; Kennedy and Heidger, 1979; Robaire and Hermo, 1988) except that the smooth endoplasmic reticulum appears to be less abundant than in these species. The abundant epithelial infoldings are supported by a highly vascular lamina propria and the network of blood vessels, like in the sperm storage region in testicond mammals (Djakiew and Caduello, 1986), may be an adaptation to increase the surface area of capillaries for the supply of oxygen for respiration of the spermatozoa residing within the lumen of the vas deferens.

In conclusion, the present study has shown that most of the spermatozoa present within the vas deferens of the spinifex hopping mouse are found in the middle and urethral segments of the duct. This region of the male reproductive tract has a highly unusual structural organization and the spermatozoa in this region retain their potential for motility even though the environmental temperature is similar to that of the core body temperature. The animals used in this study were from a...
self-sustaining captive breeding colony in which adult males have a similar reproductive anatomy to that of wild, caught individuals (Breed, 1992), thus the storage of potentially motile spermatozoa within this environment would seem to be the norm for this species. The reason why hopping mice have evolved this strategy for sperm storage is not clear at the present time but, as with some of the other unusual features of the reproductive tract of this species, it may relate to the mode of copulation which involves a physical tie, or lock, between the male and female (Dewsbury and Hodges, 1987; Breed, 1990; Breed and Washington, 1991). A comparative study of the morphology of the male reproductive tract of other Notomys species has indicated similar structure, and presumably function, of the vas deferens in *N. fuscus*, *N. mitchelli* and *N. aquilo* to that of *N. alexis*, whereas, by contrast, that of *N. cervinus* has a more typical, and presumably ancestral, structural organization with a simple narrow lumen and thick muscle coat like that of other murid rodent genera (Breed, 1986).

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